SOME OBSERVATIONS ON THE PATHOGENICITY OF B. BOTULINUS. X

GEORGE E. COLEMAN AND K. F. MEYER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

During the last four or five years, owing to the apparently increasing number of cases of food poisoning among human beings and animals definitely recognized as being due to the contamination of foods with B. botulinus, an intensive study has been undertaken of various phases of this question. More particularly is this true in the United States where, because of the increased use of home canned foods urged on the population during the World War by the government, there seem to have been more of these outbreaks. A study of the rapidly growing literature on this subject testifies to the fact that much important work has been accomplished in elucidating many of the problems, both scientific and practical, which confront those responsible for and interested in safeguarding the public health. It appears to the writers that one of the most important questions in this connection which has received insufficient attention concerns the pathogenicity of the organism itself when introduced into the animal body.

The study of this particular problem may properly be divided into three parts: (1) The injection or ingestion of detoxified B. botulinus spores or bacilli; (2) the injection or ingestion of the spores or bacilli together with a minute dose of B. botulinus toxin (or other substances) insufficient by themselves to produce symptoms of botulism; (3) the latency of injected or ingested spores of B. botulinus in the animal body where such spores, due to various causes, may later germinate, multiply or be freed of their toxin and produce symptoms of the disease.

The first of these conditions will concern us principally in this paper, though perhaps the third part is of equal importance, and being intimately connected with the first, it will be necessary to touch on its various phases from time to time. However, a more complete discussion of the last two problems will be reserved for a later paper.

Frequently spore-containing foods are eaten which may have been sufficiently heated to destroy the toxin but not the spores. As such foods may contain few or many viable spores, it becomes of the

Received for publication, Aug. 7, 1922.

gravest importance to increase our knowledge as to the possible pathogenicity of these detoxified spores thus consumed. In considering this possibility, we are immediately confronted by the following questions: Does the introduction of detoxified spores into the animal body produce symptoms or death from botulism? If so, how many such spores are necessary to produce these effects, and finally, what is the mechanism of the pathogenic manifestation of these organisms?

The work dealing with the effects of the injection of unheated B. botulinus cultures does not concern us for the moment, but a review of the few investigations that have been made with detoxified spores shows us that to produce toxic symptoms a larger number of spores is required when fed than when injected by other routes.

The early experiments of van Ermengem[1] would tend to demonstrate that "B. botulinus is absolutely incapable of reproducing itself in the animal body." His cultures of the various organs and contents of the intestinal tract were negative. He admits, however, the possibility of growth in the intestinal tract under proper, but unknown, conditions. Other European workers, following his investigations, came to similar conclusions. These workers did not use a medium in the production of their spores capable of giving a maximum toxicity. Later Dickson[2] stated that the toxin is never formed within the body, and he bases his conclusion on his belief that the toxin could not be formed at a temperature in excess of 28 C. Graham and Brückner[3] state that in cases of forage poisoning "a fatal intoxication was suggested by repeated failures to recover the organism from the blood in the course of fatal and experimental disease, while the brain, cord, liver, blood, spleen, mesentery, and suprarenals from 26 experimental cases of fed and subcutaneously injected animals were all negative."

Thom, Edmondson and Giltner[4] originally failed to produce symptoms of poisoning by feeding or injecting detoxified spores of B. botulinus, but later[5] these authors were successful. They caused death in 1 guinea-pig in 13 days, by the subcutaneous injection of only 30,000,000 spores of the Boise strain, though 31,000,000 spores of the Nevin strain produced no effect. In their feeding experiments, they produced death in 1 animal with 78,000,000 spores (Nevin strain) in 7 days, though subsequently from 75,000,000 to 112,500,000 spores of the same strain failed to produce any result. The Boise strain was fatal in from 5 to 7 days in doses of 180,000,000 to 192,000,000 spores. These authors also conducted numerous experiments based on the work of Bullock and Cramer[6] who injected calcium chloride at the same time as the spores, or at varying intervals thereafter, thereby reducing the time survival of their guinea-pigs. They admit that their results are irregular, but are convinced of the pathogenicity of the detoxified spores of B. botulinus. They also admit the possibility of growth in the animal body, as well as the absorption of intracellular toxin from the spores.

[1] Ztschr. f. Hyg. u. Infektionskr., 1897, 1, p. 26.
[2] Canad. Med. Assn. Jour., 1918, 8, p. 903.
[3] Jour. Bacteriol., 1919, 4, p. 1.
[4] Jour. Am. Med. Assn., 1919, 73, p. 907.
[5] Arch. Int. Med., 1920, 26, p. 356.
[6] Proc. Roy. Soc., London, Ser. B, 1919, 90, p. 513.

During their investigation of an outbreak of olive poisoning, Armstrong, Story and Scott[7] inoculated a guinea-pig subcutaneously with a heated suspension containing "some 300,000,000 bacilli . . . and numerous spores," with no result. Heated cultures were fed with negative results, while washed spores were fatal when force fed, but not when fed on grass or meal. These authors by experiment estimated 1 olive to contain "a minimum of 1,300,000,000 bacilli, presumably spore bearers" and believe from their work with animals that "a bite of olive containing this number of viable organisms, if capable of multiplying and forming toxin in the alimentary tract, should have caused serious infection."

Burke, Elder and Pischel,[8] from a study of numerous outbreaks of botulism in man, conclude that the ingestion of toxin free spores, particularly in small numbers, does not cause this disease. Orr[9] shows that the feeding or injection of massive doses of toxin free spores produced botulism in guinea-pigs and mice. He caused the death of 1 guinea-pig in 5 days and only "weakness" in another, by feeding 90,000,000 spores. One animal died in 33½ hours after the ingestion of 200,000,000 spores. Of 2 animals injected subcutaneously with 45,000,000 and 50,000,000 spores, the first died in 249 hours and the other in 50 hours. He believes that the spores grow in the body and produce toxin which can be demonstrated by a precipitin test, as well as by direct toxicity tests of the emulsified organs. The counted spores in some of these experiments were fed on bread, which would appear to be a source of error in the estimation of the number of spores actually entering the digestive tract. The irregularity of Orr's results is similar to that obtained by Thom, Edmondson and Giltner. Geiger, Dickson and Meyer,[10] in a complete analysis of the question of botulism, more particularly in that portion of their work devoted to its epidemiology, discuss the possible pathogenicity of the detoxified spores. They imply some doubt as to the complete detoxification of the spores used by some of the other workers who produced botulism in animals. They, themselves, obtained negative results when heavy spore emulsions were mixed with the specific antitoxin, washed, and then heated at 80 C., and they suggest that further evidence should be obtained in this matter by careful scrutiny of further outbreaks before assuming that detoxified spores naturally ingested by human beings are pathogenic. Furthermore, they cite several human cases to show that those who ate the raw spoiled food died, while those who ate the same food heated showed no symptoms. However, they add that the experience gained by a study of these outbreaks neither supports nor refutes the results of these experimental investigations as the spore content of these heated foods was unknown.

Graham, Schwarze and Boughton[11] have recently shown that freshly drawn milk from artificially infected udders of cows is, under certain conditions, lethal for guinea-pigs, but the elaboration of toxin in the animal udder tissue or mammary secretion could not be established. These authors conclude from these and numerous feeding experiments that the possibility of B. botulinus toxin being excreted in the milk of lactating animals consuming contaminating feed is remote, though the contamination of milk by the feces or other sources furnishing spores of B. botulinus is not to be disregarded.

[7] Pub. Health Repts., 1919, 34, p. 2895.
[8] Arch. Int. Med., 1921, 27, p. 265.
[9] Jour. Infect. Dis., 1922, 30, p. 118.
[10] Bull. U. S. Public Health Service, in press.
[11] Am. Jour. Pub. Health, 1922, 12, p. 659.

Several of the following factors may play an important part in estimating the value of and in comparing the results obtained by different workers along these lines. It is important to know the method of determining the number of viable spores, the approximate number of vegetating forms present, the mediums from which they were obtained, their age, the methods of freeing them from their toxin; also the time elapsed between the detoxification and counting, as well as the injection, the method employed in feeding, and finally the purity of the spores after these various manipulations. Few workers mention all of these conditions or give controls for toxicity of the culture from which the spores were obtained, or for complete detoxification at the moment of injection.

As the pathogenicity of bacteria for the animal body as a rule depends on their ability to invade and reproduce themselves in the tissues, it is unfortunate that so few workers have studied culturally the organs of experimental and clinical cases of botulism. Orr[12] recovered B. botulinus from the spleen and liver. He also found the liver and brain of his experimental animals toxic for mice, and later records a few positive findings of B. botulinus in the liver and spleen together with entirely negative results in some of his fatally infected animals injected subcutaneously and fed large doses. Dickson[13] recovered the organism from the spleens of a few of his animals injected intravenously and van Ermengem, among the first, remarked that B. botulinus could be recovered only from the organs of animals in which a large quantity of spore material had been injected by this route. The same worker,[14] as well as Ornstein[15] and Graham,[16] also reports having found B. botulinus in the spleens of fatal cases of botulism.

B. botulinus has been found by several investigators in the dejecta of animals experimentally fed with material containing toxin and spores of this organism, but we have seen no record of the finding of the toxin in the stools of clinical or experimental cases. Meyer and Geiger[17] give in detail their studies of the stools and tissues of various fatal cases of botulism, in man and in cattle. In the former, they obtained both types of the organism from the stools at varying periods after the causative meal was eaten, as well as from the wall of the jejunum in one case and from several parts of the intestine in another, but their attempts to recover the organism from the spleens in three cases were negative. The foregoing positive findings were reported, as well as "isolations from the liver, mesenteric lymph nodes, etc.," of cattle. Records and Vawter,[18] in a study of diseases of cattle report the recovery of both types of B. botulinus from infarcts of the liver.

Recently Tanner and Dack[19] published positive findings of B. botulinus in 2 out of 10 samples of stools from normal persons. Dubovsky and Meyer,[20] in a search for this organism from the stools of 45 normal persons, reported entirely negative results. In view of these results and in considering the reported positive findings from laboratories where constant work of this character is in progress, the possibility of contamination even by experienced workers should always be borne in mind.

[12] Abstr. Bacteriol., 1920, 4, p. 10.
[13] Jour. Am. Med. Assn., 1918, 71, p. 518.
[14] Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 4.
[15] Ztschr. f. Chemotherap., O., 1913, 1, p. 458.
[16] McCaskey: Am. Jour. Med. Sc., 1919, 158, p. 57.
[17] Pub. Health Rept., 1921, 36, p. 1313.
[18] Jour. Am. Med. Assn., 1922, 79, p. 132.
[19] Jour. Am. Vet. Med. Assn., 1921, 60, p. 155.
[20] Jour. Infect. Dis., 1922, 31, p. 501.

Dubovsky and Meyer,[20] in a series of human cases seem to have taken cultures from more of the organs than we have found reported elsewhere—they studied the spleen, liver, brain, lungs, kidneys and wall of the jejunum, ileum and colon, besides the intestinal contents. In 4 cases studied they found B. botulinus, types A and B, in the intestinal contents, and in various parts of the wall itself in cases from the outbreak at Florence, Arizona; and once in the liver and intestines of one case in the Healdsburg outbreak, though the findings for the other organs were negative. Their direct toxin tests from all this material also gave negative results. The same authors also obtained cultures from the organs and intestinal contents of horses, cattle and chickens, as well as from the crops of the latter. While complete findings are not given, they mention having recovered the organism from the spleens and livers of chickens, also frequently from their crops and intestines. The livers and mesenteric lymph nodes from 2 cows were positive. They admit in their article, which embraced work undertaken over a year ago, that it had not been proved that there is an etiologic relationship of these organisms to the lesions or organs from which they have been isolated and comment on the significance of the findings from the crops and intestinal contents of chickens which swallow large quantities of soil. In the light of these findings, Dickson's failure to recover B. botulinus from the colon and ileum of 250 grain fed hogs does not seem remarkable, providing these hogs were not fed other foods which might have been grossly contaminated with spores of B. botulinus. It is possible, however, that these negative results are due to the taking of cultures from insufficient fecal material, as the work of this laboratory along these lines has shown that occasionally these spores may be found in the feces of normal guinea-pigs and rabbits as well as in grain.

The records of the study of the pathology of the various organs in human beings and animals which have shown symptoms of botulism poisoning are even more meager than the reports of cultural studies. A few European workers and Wilbur and Ophüls,[21] Armstrong, Story and Scott,[6] Meyer and Geiger,[17] and Dickson[22] in this country, mention the histologic changes found. Most of these authors noted a characteristic type of thrombus formation in the vessels of the brain. Dickson[23] believes that these thrombotic changes are not responsible for the symptoms of botulism, as they are rarely found in animals which have died within 48 hours after receiving an injection. In a recent important communication, Warthin[24] describes not only the lesions encountered in the brains of 6 human cases studied, but remarks that "all presented similar features of antemortem gas production in the brain and formation of small gas cysts containing bacilli morphologically resembling B. botulinus. These cases present evidence that human botulism is an infection as well as an intoxication."

Some of the negative results in the search for B. botulinus in the organs by the various workers may be due to the cultural methods employed, to the delay in taking cultures or previous chemical treatment of necropsy material from human cases. However, an analysis of the results obtained as well as a consideration of the opinions expressed

[21] Arch. Int. Med., 1914, 14, p. 589.
[22] Monograph, Rockefeller Institute, No. 8, 1918.
[23] Jour. Am. Med. Assn., 1921, 77, p. 483.
[24] Ibid., 1922, 79, p. 71.

shows an increasing probability that further study along these lines will definitely decide the question as to the growth of B. botulinus in the animal body.

TECHNIC

The spores of B. botulinus used were obtained from cultures in veal peptic digest gelatine, from 2 to 4 months old. They were washed from 1 to 3 times in 60 to 75 c c of saline phosphate, suspended in the same medium, heated 1 hour at 80 C. in a sealed tube at the same time as the supernatant culture fluid and final wash water, and injected or fed immediately thereafter. Guinea-pigs were used in our experiments unless otherwise stated. They were usually fed while fasting and invariably with 1 c c of suspension by means of a pipet attached to a rubber tube held in the operator's mouth. As a control for toxicity of the culture from which the spores were obtained and for the detoxification of the spores themselves, both heated and unheated supernatant culture fluid and heated and unheated final wash water were injected subcutaneously into guinea-pigs. This precaution was always taken and, in order to save repetition, the protocols of these injections for each experiment will not be given. Exceptions to this technic are to be noted in the experimental data given in tables 1 and 2.

Direct microscopic spore counts checked by cultivated dilutions in both beef heart and agar were made immediately after heating and the approximate number of vegetative forms noted. The purity of the spores was verified later from the cultivated dilutions.

A rather full consideration of the technic employed in the necropsies and taking of culture from the organs of our animals may not be out of place in view of the negative results obtained by the early workers and the paucity of records of positive findings of the organism in the various organs by recent investigators.

Guinea-Pigs.—It is essential to use animals from a healthy stock in order to avoid unobserved symptoms or death from an intercurrent infection, with consequent distribution of various contaminating organisms throughout the body. As it is possible, where B. botulinus is a common soil anaerobe or when the guinea-pigs have been kept in cages possibly grossly contaminated with B. botulinus, to find this organism occasionally in the feces and rarely in the organs of apparently normal animals, it is imperative that each guinea-pig used should be from strictly fresh stock and placed and maintained separately in small cages which have been adequately sterilized. This we have done.

Necropsy.—Instruments have been sterilized in paraffin oil for 10 minutes at 150 C., cooled and, when not in use, kept covered with a sterile towel throughout the operation. The guinea-pigs have been completely immersed from 1½ to 2 minutes in paraffin oil at the temperature noted, drained for a moment and placed on a thoroughly flamed necropsy board. Sometimes, particularly during necropsy of our controls, a sterile towel also has been laid on the board. Separate instruments have been used in the handling of each organ. The pleural and abdominal cavities have been opened separately to avoid the spread of blood. The assistance of an aide and rapid work greatly minimize the opportunities for contamination. In spite of the most rigorous precautions, some contaminations probably occurred from the air or were originally present in the organs of the animal.

TABLE 1

RESULTS OF FIRST EXPERIMENTS, PERFORMED WITH STRAIN 97, TYPE A — MANY RODS

No.	Guinea-Pig Weight, Gm.	Dose	Product	Symptoms	Survival	Positive Organs	Remarks
Toxic Spore Suspension, Subcutaneous							
1	300	0.1 c c	Supernatant toxin	Botulism	Died after 45 hrs.		
2	300	0.1 c c	Supernatant toxin heated 1 hr. at 80 F.	.....	Survived	.....	Centrifugalized 1½ hrs.
3	280	1.0 c c	Second wash before heating	Botulism	Died after 21 hrs.	.....	Centrifugalized ½ hr.
4	290	1.0 c c	Second wash after heating	Botulism	Died after 5 days	Spleen, mes. liver, blood bone marrow (mice ++)	Centrifugalized ½ hr.
5	280	2.0 c c	Second wash after heating	Botulism	Died after 45 hrs.	.....	Centrifugalized ½ hr.
Spore Injections, Subcutaneous (Dose, 2 c c)							
7	244	1,120 B	.....	Botulism	Died after 24 hrs.	Spleen, liver (mice ++)	
8	247	1,120 B	.....	Botulism	Died after 20½ hrs.	Blood, Spleen, Liver, Stomach } mice +	
9	279	560 M	.....	Botulism	Died after 24 hrs.	Bonemarrow, Mes., Liver, Spleen } mice +	
10	258	560 M	.....	Botulism	Died after 24 hrs.	Liver, spleen blood mouse ++	
12	330	112 M	.....	Botulism	Died after 4 days		
13	345	112 M	.....	Botulism	Killed after 7 days, moribund	Liver, Brain, Ing., Spleen } mice +	
14	308	11.2 M	.....	Not botulism	Died after 18 days	.....	General infection, gained weight
15	395	11.2 M	.....	None	Survived		
17	320	1,120 M	.....	None	Survived		
18	265	1,120 M	.....	None	Survived		
Fed Spores (Fed While Fasting)							
20	310	2,240 B	.....	Botulism 7 days	Killed after 8 days	Blood, spleen, mes., liver	All mouse +
21	410	2,240 B	.....	Botulism 7 days	Killed after 8 days	Liver, spleen, mes., R. bone	All mouse +
22	380	1,120 B	.....	None, lost weight	Killed after 10 days	Lung, bone, liver	All mouse +
23	320	1,120 B	.....	None, lost weight	Killed after 10 days	Mes., bone, spleen, ing., liver	All mouse +
24	250	560 M	....	None, gained weight	Killed after 15 days	Bone, lung, spleen, ing., liver, feces	Feces mouse +
25	250	560 M	.....	None, gained weight	Killed after 15 days	Ing., feces	Feces mouse +
26	240	280 M	.....	None, gained weight	Killed after 16 days	Inguinal lymph-node	
27	240	280 M	.....	None, gained weight	Killed after 16 days	Liver, lungs	
29	250	140 M	.....	None, gained weight	Survived		
30	280	140 M	.....	None, gained weight	Survived		
31	250	70 M	.....	None, gained weight	Survived		
32	250	70 M	.....	None, gained weight	Survived		
19	350	Check	.....	Lost weight	Died after 19 days	Ing., kidney (mice ++)	Intercurrent infection

Ing., inguinal lymphnode; Mes., mesenteric lymphnode; Bone, bonemarrow; B, billion; M, million.

It is to be borne in mind that the subsequent use of the word contaminated as applied to the culture of organs does not necessarily imply contaminations from without. In cases in which organisms so closely associated culturally and morphologically as B. botulinus and B. sporogenes, pathogenic and slightly pathogenic, are growing together in the intestinal tract, it is not unreasonable to suppose that the invasion of the organs by the former may carry through certain individuals of the other species. Heller[25] mentions that while B. sporogenes is not invasive in small doses in pure culture, it may invade the tissues in company with other organisms. This appears to be true for other anaerobes as well. Also since it has been recently stated[26] that the gallbladder and its ducts in 63% of human cases brought to operation contained bacteria, it is not surprising occasionally to find invasive organisms other than B. botulinus in the organs of our guinea-pigs. Stress is laid on these facts in order that the thoroughness of our technic may not be questioned.

Cultures.—The beef heart and liver agar mediums and the reasons for their use in the botulism work of this laboratory have already been fully discussed by Dubovsky and Meyer.[20] As the final determination of the presence of B. botulinus in cultures, unless antitoxin neutralization tests are to be made, depends on the heat resistance of its spores, we have found it desirable to use this medium.

The organs were placed in the freshly boiled and cooled beef heart medium and incubated from 7 to 15 days at 35 C. and reincubated when necessary. All cultures, regardless of appearance or lack of odor, were examined microscopically, and material from those tubes which showed sporulating organisms in any way morphologically resembling B. botulinus or free spores with or without bacilli, were inoculated into deep agar, heated from 1 to 1½ minutes at 100 C., incubated from 1 to 7 days at 35 C., and the character of the colonies observed. When such colonies or a sufficient number of them were doubtfully positive for B. botulinus, the original beef heart culture was inoculated into mice. In many apparenlty sterile beef heart cultures a long search will result in the finding of an occasional free spore where nothing else is seen. These spores may or may not prove to be viable. In contaminated cultures, more particularly in those in which the contaminating organisms are cocci or anaerobes of the B. bifermentans or B. sporogenes types, the latter may almost completely overgrow the few organisms or spores of B. botulinus originally in the culture, as has been explained by Reddish.[27] In this paper, he also mentions the possibility of an agar colony of B. sporogenes containing within its body spores of B. botulinus. The impossibility of seeing these, as well as the failure to recognize the atypical colonies of B. botulinus, may have caused us to give some erroneously negative findings. Considering the foregoing as well as other sources of error, and in spite of the precautions taken, we believe that we have sometimes failed to show the presence of B. botulinus spores in the organs of our experimental animals and that the results given in our tables are possibly too low. However, we wish to state that in the organs of our controls every possible method was used to demonstrate the presence of the organism.

Cultures of the following organs were always made: Blood, bone marrow, inguinal and mesenteric lymph nodes, liver and spleen, and often the brain, lungs, kidneys and sometimes the feces and thigh muscle and gallbladder. The results of our experiments are now presented.

[25] Jour. Bacteriol., Jan. 1922, 7, p. 29.
[26] Beitr. z. klin. Chirurg., 1922, 125, p. 377.
[27] Jour. Infect. Dis., 1921, 29, p. 126.

EXPERIMENTAL DATA

Our preliminary experiments (tables 1 and 2) do not conform in all respects to the description of our technic, but as the results have an important bearing on the subject to be discussed later, we have decided to include them. The spore suspension in the first experiment and the heated and unheated toxin and final wash water (table 1) were ready too late on a Saturday afternoon to inject and so were left in the icebox until the following Monday morning (about 40 hours), when they were injected. They proved to be toxic.

TABLE 2

RESULTS OF EXPERIMENTS WITH STRAIN 38, TYPE A—FEW VEGETATIVE FORMS

Toxic Spore Emulsion; Final Heated Wash Water (Icebox 48 Hrs.) Subcutaneous

No.	Guinea-Pig Weight, Gm.	Dose	Symptoms	Survival	Positive Organs	Remarks
52	178	2 c c	Botulism	Died after 9½ days	All but brain	
53	170	2 c c	Botulism	Died after 4 days	Liver, spleen	
54	158	1 c c	Botulism	Died after 12 days		
55	150	1 c c	Botulism	Died after 11 days		
			Subcutaneous Spore Injections			
56	294	1.8 B	Botulism	Died after 20 hrs.	All + but ing. and brain	
57	248	1.8 B	Botulism	Died after 5½ days	Brain, liver, bone, mes., blood	
58	255	900 M	Died in night	Died after 18 hrs.	Ing., blood, mes., spleen bone	
59	240	900 M	Botulism	Killed after 3 days	Mes., spleen, liver, bone	
60	199	180 M	Botulism	Died after 14 days		
61	222	180 M	None	Survived		
62	230	Check	None	Killed after 16 days	Bone (mouse +) all other organs negative	
63	205	18 M	Indefinite	Died after 12 days	.....	Intercurrent infection
64	202	18 M	Indefinite	Died after 13 days	.....	Intercurrent infection
65	217	1.8 M	Indefinite	Died after 7 days	.....	Intercurrent infection
66	203	1.8 M	Indefinite	Survived		
			Fed Spores (Fed While Fasting)			
67	220	3.3 B	Botulism	Died after 4½ days	Ing., liver, stomach	
68	300	3.3 B	None	Killed after 10 days	Ing., liver	
69	200	1.650 B	None	Survived		
70	190	1.650 B	None	Survived		
72	165	1.250 B	None	Survived		
73	183	1.250 B	None	Survived		
74	181	725 M	None	Survived		
75	222	725 M	None	Survived		
	240	Check	None	Killed after 16 days	All organs negative	

As these results were so at variance with those of previous workers, even though the wash water containing few spores and consequently the spore suspension (afterward diluted) were toxic, we decided to repeat this experiment under similar conditions with a portion of the emulsion used in the experiment summarized in table 3.

In this duplicated experiment, the heated spore suspension and final heated wash water were left in the icebox for 48 hours (Table 2).

These two experiments show the necessity of injecting spore suspensions immediately after heating.

The result of our next experiment, in which the spores were completely detoxified, is given in table 3.

As will be seen from a comparison of the data given in the foregoing tables, an enormous number of B. botulinus spores are necessary to produce symptoms and death, especially when fed to guinea-pigs.

As we were able to cause symptoms or death in our guinea-pigs only by the injection and feeding of spores of B. botulinus in doses considerably in excess of those employed by other investigators, and as we found a similar irregularity in our results, we thought it would be of interest to ascertain whether it were possible to break the apparent immunity of some of our animals to large doses. Several of these guinea-pigs had survived without presenting symptoms, while others receiving the same dose succumbed.

It has long been known that the spores of pathogenic bacteria, when freed from their toxin, may remain latent in the tissues of the body for considerable periods, and when anything happens, such as slight localized infections or prophylactic vaccinations, to upset the equilibrium of the bodily defenses or to break down its resistance, such spores may germinate and cause infection. An intensive study of this question has been undertaken by different investigators.

Koser and McClelland[28] have shown that while it is unusual to recover the spores of the aerobes away from the site of inoculation, the spores of the anaerobes may be recovered from the different organs up to 4 months after inoculation. They confirm the results of many others, especially as regards B. tetani, which has been found in most of the organs of the body in fatal human cases, as well as in experimental animals. Canfora[29] recovered free spores of B. tetani from the blood stream as early as 12 hours and up to the seventieth day after subcutaneous injection. Vaillard and Vincent,[30] working with lactic acid and B. prodigiosus, Massart and Bordet,[31] with lactic acid,

[28] Jour. Med. Res., 1917, 37, p. 259.
[29] Centralbl. f. Bacteriol., I, O., 1908, 45, 495.
[30] Ann. de l'Inst. Pasteur, 1891, 5, p. 1.
[31] Ibid., 1891, 5, p. 417.

Semple,[32] with quinine, and Teale and Bach,[33] with lactic acid, sodium bicarbonate, calcium chloride and other substances, have all been able, under varying conditions, to break down the resistance of the host to the spores of B. tetani or of other bacteria by the injection of these substances. The latter workers believe from their experiments that, while sometimes the spores

TABLE 3

RESULTS OF EXPERIMENTS WITH STRAIN 38, TYPE A — FEW VEGETATIVE FORMS

Nontoxic Spore Emulsions; Injected Immediately After Heating

No.	Guinea-Pig Weight, Gm.	Dose	Product	Symptoms	Survival	Positive Organs	Remarks
34	410	0.1 c c	Supernatant toxin	Botulism	Died after 24 hrs.		
35	360	1.0 c c	Supernatant toxin 1 hr. at 80 C.	None	Survived		
36	357	1.0 c c	Second water before heating	Botulism	Died after 24 hrs.		
37	245	1.0 c c	Second water after heating	None	Survived		
39	300	1.0 c c	Second water after heating	None	Survived		
40	278	1.0 c c	Second water after heating	None	Died after 14 days	.....	Intercurrent infection
41	270	2.0 c c	Second water after heating	None	Survived		
42	270	2.0 c c	Second water after heating	None	Survived		
43	250	2.0 c c	Second water after heating	None	Survived		
			Fed Spores (Fed While Fasting)				
45	275	11 B	.....	Botulism 60 hrs.	Died after 96 hrs.	All + except ing.	Brain not cultured
46	272	11 B	.....	Botulism 22 hrs.	Died after 60 hrs.	All +	Lung, kidney not cultured
47	242	5.5 B	.....	Botulism 60 hrs.	Killed after 71 hrs., moribund	Liver, kidney, bone, brain, blood, mes.	
48	210	2.75 B	.....	None	Survived		
49	229	5.5 B	.....	None	Killed after 14 days	Ing.	
50	192	2.75 B	.....	None, lost 40 gm.	Died after 14 days	All + except lungs	
C	350	Check	.....	None	Survived		

do not germinate, at other times they do, but that they do not appear to multiply in the tissues. In many of the different experiments cited in the foregoing, cultures of the organs gave positive findings for the spores of the species of organism injected. Tulloch[34] injected 1,000,000,000 toxin-free spores

[32] Scient. Mem. Officers and San. Dept. Gov't. India, 1911, No. 43, new series (cited by Koser and McClelland).

[33] Jour. Path., 1920, 23, p. 315.

[34] Brit. Med. Jour., 1918, 1, p. 614.

of B. tetani into animals without producing the corresponding disease. The subsequent inoculation of B. welchii toxin caused the germination of the spores of B. tetani. The experimental data summarized in tables 1 and 2 tend to confirm to a certain extent the work of Tulloch as to the accessory action of a toxin, in this case homologous, more especially when the toxic spores were subcutaneously administered. As we were able to produce botulism by feeding toxic spores only in excess of 1,120,000,000 in one case and in excess of 1,650,000,000 in another, it is possible that, due to various factors, absorption of a slight amount of toxin from the intestinal tract is prevented. This phase of the question will be referred to later. When the results of these tables are compared with those recording our experiments with non-toxic spores (table 3) and those to be given later, the differences between our results, especially in subcutaneous injections with toxic and toxin-free spores and those of other workers is apparent. The observations of Geiger, Dickson and Meyer[10] as to the difficulty of detoxifying spores by washing or slight heating at 80 C., as many of these investigators have done, are borne out, and this may be one of the causes of the discrepancies between their results and ours. The cultural findings in guinea-pig 52 (table 2) which received toxic wash water subcutaneously is interesting. While the injection of this water would probably have caused a fatal outcome in this case had it not contained very few spores, its toxicity would appear to have been instrumental in causing the spread of these spores, or the bacilli possibly arising from them, throughout all the organs of the body except the brain. This does not seem to occur even in subcutaneous injection unless many millions of detoxified spores are introduced. Here, again, we probably have a confirmation of Tulloch's findings.

Bullock and Cramer[6] and Edmondson, Giltner and Thom[5] have shown that calcium chloride injected at the same time or within a few minutes after the subcutaneous injection of pathogenic spores, the latter working with B. botulinus, greatly diminishes the time necessary to produce symptoms or death. The latter workers showed that such injections produced no effect if given two days after the spore injection. Their animals were all injected subcutaneously both times.

Though Teale and Bach[33] had fewer failures by the intraperitoneal route, our injections were generally made subcutaneously within 1 to 2 cm. of the site of the original injection. Proper controls for each substance were also made. In view of the results of the latter workers, we are not altogether surprised at our failure by this means, after the interval shown in our tables, to alter the effect of the spores when either fed or injected. Our cultural studies of the organs of other animals lead us to suppose that the tissues of the guinea-pigs thus injected had already been invaded by the spores or bacilli at the time of the injection of such additional substances, and any negative chemotactic property these substances may have possessed was useless at the time they were injected, so that this failure must be attributed to a different cause. Tables 4, 5 and 6 show our experiments in detail.

During the course of these experiments, we endeavored to determine how rapidly the spores of B. botulinus leave the site of subcutaneous and intraperitoneal injections. We inoculated 2 c c of a suspension of detoxified spores containing a few bacilli, some of which colored partially by the Gram method, into the peritoneum of 2 guinea-pigs, one of which had been previously

TABLE 4

RESULTS OF EXPERIMENTS WITH STRAIN 58, TYPE A — VERY FEW RODS

Subcutaneous Injections

No.	Guinea-Pig Weight. Gm.	Spores	Symptoms	Survival	Organs Positive	Remarks
80	269	504 M	Botulism	Died after 15 days	All organs + spores in liver	
81	275	504 M	None	Killed after 25 days	Spleen, liver, mes., ing.	After 9 days received 0.5 c c N. Na_2CO_3 intraperitoneally, no symptoms
82	254	252 M	None	Died after 15 days	All organs + spores in liver	
83	227	252 M	None	Killed after 21 days	Liver, bone, ing.	After 9 days received 0.5 c c N. Na_2CO_3 intraperitoneally, no symptoms
84	225	42 M	None	Killed after 24 days	Liver, spleen, kidney, bone, mes.	After 8 days received same as above
85	214	42 M	None	Survived	.....	After 14 days received 0.5 c c N. lactic acid intraperitoneally, no symptoms
86	214	Check	None	Survived		

Fed Spores (Fed While Fasting)

87	223	Check	None	Survived	.....	Necropsy, table 6
88	300	2.520 B	None	Killed after 10 days, gained 60 gm.	Liver, brain, L. bone	
89	296	2.520 B	Typical botulism 7 days	Killed after 8 days, moribund	Lungs	
90	288	1.008 B	None	Killed after 24 days	Ing., feces	
91	280	1.008 B	None	Survived	.....	After 13 days fed 0.00675 gm. quinin sulphate, no symptoms
92	259	504 M	None	Survived		
93	258	504 M	None	Killed after 27 days, gained 118 gm.	Bone, feces +	
94	251	252 M	None	Killed after 24 days, gained 92 gm.	All negative, feces +	
95	248	252 M	None	Survived	.....	After 9 days received 0.5 c c N. lactic acid intraperitoneally, no symptoms
96	243	Check	None	Survived	All negative	
97	242	1.176 B	None	Killed after 21 days	Liver	After 14 days received 0.005 gm. quinin sulphate subcutaneously, no symptoms

injected in the same manner with 5 c c of sterile broth. At 15 minute intervals after injection of the spores, a few drops of fluid were withdrawn from each animal and stained by the Gram method on a slide containing a smear from the spore suspension inoculated. No microscopic differences were apparent in preparations from the 2 guinea-pigs. The leukocytes englobed the

spores and bacilli, and at the end of 6 hours they had practically disappeared from the site of inoculation. Three guinea-pigs were injected subcutaneously with 2 c c of an emulsion of detoxified spores, a pocket having been made under the skin with the needle of the syringe. One guinea-pig also received mixed with the spores, 1 c c of a 2% solution of calcium chloride and another, 1 c c of B. botulinus antitoxin added to the spores. The death of the guinea-pig receiving calcium chloride preceded that of the control by a long period of time. Again no differences were noted in the stained preparations after varying intervals up to 24 hours. In the beginning, few leukocytes were present, but they gradually appeared until at the end of 24 hours an intense leukocytosis was present. The disappearance of the spores and bacilli was rapid, and at the end of 5¾ hours few were microscopically visible, either free or in the leukocytes. A few rare leukocytes at this time were gorged with spores and bacilli both in the guinea-pig receiving antitoxin and in the one receiving calcium chloride. Saline washings from these pockets were nontoxic for mice. While it is more than likely that some of the injected spores germinated in these pockets or in the peritoneum within 24 hours, we have no reliable evidence that this was the case. There were not sufficient differences between the Gram stained preparations from the animal fluids and the original spore material on the same slide to warrant a definite statement that this is so. We have sometimes noted a few gram-positive organisms in our heated spore suspension, so that unless there is marked growth or additional evidence is obtained that these rare bacilli seen within a short time after injection are B. botulinus, one should accept with reserve these findings as evidence of growth. The result with calcium chloride is not in accord with that of Thom, Edmondson and Giltner,[5] who found that the leukocytes did not englobe the bacilli and presumably also the spores of B. botulinus when injected with this substance. These authors remark that smears showed a few bacilli in the exudate from these subcutaneously injected guinea-pigs and that "cultures revealed the presence of the bacilli in virulent form," but they do not state how they determined that their cultures originated from bacilli rather than from spores. They suggest from the presence of bacilli on their smears that growth of B. botulinus must have occurred in the animal body, but they do not mention any controls of colorations of the spore material injected, nor do they state the method used to color their smears nor describe the bacilli seen. Their results are interesting but need amplification.

Teale and Bach[33] have stated that they failed to prevent the phagocytosis of bacteria by injecting lactic acid simultaneously with the bacteria. These authors discuss at length the question of latency of anaerobic organisms and believe that the spores germinate and the vegetative forms thus arising remain latent. Whatever may be the mode of action of these accessory factors in accelerating the appearance of B. botulinus symptoms such action would not seem to be due entirely to any negative chemotactic property which such substances may possess for the leukocytes or other phagocytes.

Several of the authors previously quoted have considered that the pathogenicity of the detoxified spores and killed vegetative forms might be due to the action of the enzyme of the blood or tissues in liberating whatever preformed toxin they might contain. While this possibility, for various reasons, seems to us exceedingly remote, we thought it worth while to try the following experiment: Emulsions of counted spores of 2 strains and 1 other in which the number of spores was unknown were heated in sealed tubes at 122 to 125 C. for 5 minutes. The tube in which the spores were uncounted

TABLE 5

RESULTS OF EXPERIMENTS WITH STRAIN 97, TYPE A — MODERATE NUMBER RODS; FEEDING EXPERIMENTS

Guinea-Pigs

No.	Weight	Product	Symptoms	Survival	Positive Organs	Remarks
98	450	5.08 B + ½ c c N. serum subcutaneously	None, lost 66 gm.	Killed after 30 days	Lungs, brain, feces	After 10 days received 0.005 gm. quinin sulphate subcutaneously, no symptoms
99	420	5.08 B + 1 c c antitoxin A subcutaneously	None, gained 10 gm.	Killed after 30 days	Lungs	
100	410	5.08 B + 1 c c N. serum subcutaneously	Not botulism, lost 120 gm.	Died after 5 days	Liver	Paratyphoid
101	354	5.08 B + 1 c c antitoxin A subcutaneously	Not botulism, lost 29 gm.	Died after 3 days	Brain, liver, L. bone	General infection
102	259	Check	Not botulism	Died after 13 days	All organs negative	

Mice

1	Medium size	1.27 B + ½ c c N. horse serum subcutaneously	None	Killed after 10 days	No cultures	
2	Medium size	1.27 B + ½ c c antitoxin A subcutaneously	None	Killed after 10 days	Liver, spleen, bone	
3	Medium size	1.27 B + ½ c c N. serum subcutaneously	None	Killed after 10 days	Blood, bone	
4	Medium size	1.27 B + ½ c c antitoxin A subcutaneously	Indefinite	Died after 2½ days	Liver, spleen	
5	Medium size	Check	.....	Killed after 10 days	All negative	

Rabbits

	2,600	63.5 B	None until 31st day dysentery, gained 200 gm. to 20th day	Killed after 35 days	Bone, ing., feces	After 31 days, fed 25,000 M.L.D. filtered toxin; no symptoms except continuation of dysentery

and which contained enormous numbers was immediately opened, 3 drops inoculated into beef heart and the remainder (about 2 c c) injected into a guinea-pig. The other 2 tubes, which contained 17,000,000,000 and 20,000,000,000 spores, respectively, were placed in the incubator and icebox alternately for several hours during 6 days. They were then opened, inoculations made into beef heart and the remaining contents centrifugalized. The deposit and super-

TABLE 6

RESULTS OF EXPERIMENTS WITH STRAIN 62, TYPE A — RARE VEGETATING FORMS

Subcutaneous Injections

No.	Weight	Dose	Symptoms	Survival	Organs Positive	Remarks
109	252	1.459 B	Botulism	Killed after 4 days, moribund	Mes., liver	
110	250	1.459 B	Botulism 24 hrs.	Died after 43 hrs.	All organs +	
111	247	230 M	None	Killed after 20 days	Liver, spleen, ing., bone, mes.	
112	232	230 M	None	Killed after 20 days	Ing. (mouse +)	After 23½ hrs. received 0.9 c c 2% N. $CaCl_2$ subcutaneously, no symptoms
113	207	230 M	None, 21 days gained 45 gm.	Survived		
114	200	230 M	Botulism	Died after 4½ days	Ing., bone, spleen, liver, brain, blood	After 23½ hrs. received 0.9 c c 2% N. $CaCl_2$ subcutaneously, no symptoms
115	285	Check	None	Survived		

Fed Spores (Fed While Fasting)

117	320	11.5 B	Botulism 3 days	Killed after 4½ days moribund	Bone, brain, liver, feces	Lost 73 gm.
118	289	11.5 B	Indefinite	Died after 3½ days	Mes.	Lost 29 gm.
119	286	5.75 B	Botulism 6 days	Died after 13 days	Liver, feces	Lost 126 gm.
120	282	5.75 B	Botulism	Died after 6½ days	Liver, bone, brain	Lost 100 gm.
121	270	2.875 B	None	Survived	.....	Released after 5 wks.
122	270	2.875 B	None	Killed after 15 days	Lungs, brain, feces	Gained 40 gm.
123	260	1.150 B	None	Killed after 15 days	All negative	Gained 52 gm.
124	258	1.150 B	None	Killed after 15 days	All negative	After 22¼ hrs. received 0.9 c c 2% N. $CaCl_2$ subcutaneously, no symptoms, gained 79 gm.
125	177	1.150 B	Indefinite	Died after 4 days	Liver, feces	Lost 14 gm.
126	200	1.150 B	Lost 67 gm.	Died after 16 days	All negative, feces +	After 22¼ hrs. received 0.9 c c 2% N. $CaCl_2$ subcutaneously, no symptoms
87	330	Check	None	Killed after 16 days	Liver, feces (mice ++)	Gained 50 gm.

natant fluid from each tube (about 1.5 c c) were injected separately under the skin of different guinea-pigs. None of these injections produced symptoms, showing that the protein substance, or its autolyzed products of killed B. botulinus spores and rare bacilli in the amounts injected is not toxic when introduced subcutaneously into guinea-pigs. The inoculated mediums from all these suspensions remained sterile.

Though the wall of a viable spore may be highly resistant to heat, we know that when subjected to a temperature of 80 C. for 1 hour the protein contents undergo some damaging changes, if not a certain degree of coagulation, as often evidenced by greatly delayed ability to germinate after being heated at this temperature. Also, it probably could be shown by quantitative cultural studies before and after heating to 80 C., as has been done for a temperature of 100 C., that many of the least resistant spores had been killed at this temperature. That is why we have always checked our microscopic counts by cultivated dilutions after heating. Since the temperature at 80 C. produces some of these vital changes, it is more than likely that it destroys the intracellular toxin of the spores as well.

A summary of the toxicity of the different strains employed in the foregoing experiments, as well as the minimum dose required to produce definite symptoms of botulism is given in table 7.

TABLE 7

TOXICITY OF STRAINS

Strain	Toxic Spore Emulsion	Minimum Dose Given Subcutaneously	Death from Botulism	Minimum Dose Fed	Death from Botulism	Remarks
97	Yes	112 M	4 and 7 days	2.24 B	Killed after 8 days	Very ill; rats killed
97	No	.....	.....	5.08 B	2 survived	1 received antitoxin
97	No	.....	.....	5.08 B	.....	2 died, after 3 and 5 days, botulism very doubtful; 1 received antitoxin
97	No	.....	.....	63.5 B	(Rabbit) survived	Killed after 35 days
38	No	.....	.....	2.75 B	14 days, 1 survived	
38	Yes	180 M	14 days, 1 survived	3.3 B	4½ days, 1 survived	
58	No	252 M	15 days, 1 survived	2.5 B	7 days, 1 survived	
62	No	250 M	4½ days, 3 survived	5.75 B	13 days, 6½ days	

From table 7 it will be seen that strain 58 was the most toxic in the feeding experiments, while strain 38 appears to be the most toxic of those injected subcutaneously. However, due to the few experiments recorded with each strain, the individual susceptibility of the animals or other unknown causes, no definite conclusions can be drawn as to any differences which may exist in the pathogenicity of the spores of each strain. Owing to the fact that many of our guinea-pigs were killed with a view to the study of the organs, perhaps some of these might have developed symptoms had they been allowed to live longer. For this reason, we do not categorically deny the possibility of producing botulism for these strains in doses below those given in table 7, but it would appear that these strains, in our hands, all of which produced culturally a potent toxin, were not pathogenic in small doses like those recorded by other workers for other strains.

A summary of the cultural studies of the various organs of our experimental animals, as shown in the tabulation given in the foregoing, is given in tables 8 and 9. In figuring the percentages of the results of this work, we have included cultures of the organs from a few animals (intraperitoneal and subcutaneous leukocyte tests) that received detoxified spores but which are not included in our tabulated results.

While, as we have said before, we do not believe that we have been successful, especially in our earlier experiments, in demonstrating B. botulinus in all of the organs when it has been present, the main fact to be deduced from these results is the invasion of all the organs of

TABLE 8

RESULTS OF CULTURAL STUDIES OF VARIOUS ORGANS OF EXPERIMENTAL ANIMALS

Organ	Subcutaneous						Fed						Total + Died or Dying of Botulism
	Died or Dying		% +	Killed, No Symptoms		% +	Died or Dying		% +	Killed, No Symptoms		% +	
	+	—		+	—		+	—		+	—		
Ing. lymph.....	5	12	30	3	3	50	4	14	22	4	17	19	25%
Bone.....	11	6	64	2	4	33	8	10	44	8	13	40	54%
Blood (heart)..	9	8	53	0	6	0	2	16	10	0	21	0	31%
Mes. lymph.....	10	7	59	2	4	33	7	11	40	1	20	5	50%
Spleen.....	13	4	77	2	4	33	6	12	33	3	18	14	54%
Liver.....	16	1	94	3	3	50	14	4	80	8	13	38	86%
Kidney.....	5	1	83*	1	0	—	4	9	32*	0	9	0	48%*
Brain.....	6	2	75*	0	4	0	7	6	54*	3	14	12	60%*
Lungs.....	2	1	66*	1	1	—	3	5	37*	8	9	47	45%*
Muscle.....	0	1	0	0	0	0	0	2	0	0	1	0	0
Feces.....	0	0	0	0	0	0	3	0	100*	11	2	91*	100%*
Stomach†													
Total guinea-pigs.....	17			6			18			21			62

* Percentage of times cultures were taken from organ.
† Intraperitoneal 2 guinea-pigs, all + except blood.

the body regardless of the mode of administration of the spores. In those animals in which a subcutaneous injection of spores produced a fatal result, the organism was recovered more often than in those that died after the spores were fed. In the foregoing experiments the few microscopic examinations of smears of these organs, or of the heart blood, which we have made have been negative, with the exception of a few cases in which we have seen rare spores or bacilli in the liver and blood. Undoubtedly enormous numbers of the spores are eliminated by the leukocytes of the blood or by the endothelial cells of the tissues, especially of the liver and spleen.

To what extent the spores and bacilli of B. botulinus are actually destroyed and where is problematical. The literature on this subject is filled with interesting discussions based on intravenous injections

into animals of inert substances and bacteria. Drinker and Shaw [35] Fenn,[36] Nagao [37] and numerous others working with manganese dioxide, carbon, India ink, etc., and Nagao [38] using streptococci, as well as Meyer, Neilson and Feusier,[39] who injected typhoid bacilli, have made a thorough investigation into the fate of these organisms when

TABLE 9

ANIMALS KILLED AFTER SPORE INJECTIONS

Subcutaneous, No Symptoms nor Lost Weight

Days	Liver	Spleen	Bone	Mes.	Ing.	Brain	Kidney	Lungs	Feces
20	+	+	+	+	+	..	..	..	..
20	..	..	..	..	+	..	..	..	..
21	+	..	+	..	+	..	..	..	..
24	+	+	+	+	..	..	+	..	..
25	+	+	..	+	+	..	..	..	..
	4	3	3	3	4	0	1	0	
Fed, No Symptoms Nor Lost Weight									
10	+	..	..	..	+	..	..	..	0
10	+	..	+	..	..	+	..	..	..
10	+	+	+	..	..	..	..	..	0 Mice
10	..	..	+	..	..	..	..	..	0 Mice
14	..	..	.	..	+	..	..	..	0
15	+	+	+	..	..	..	..	+	+
15	..	..	..	..	+	..	..	..	+
15	..	..	..	..	..	+	..	+	+
15	0	0	0	0	0	0	0	0	+
15	0	0	0	0	0	0	0	0	+
16	+	..	..	..	..	..	..	+	0
16	+	..	..	..	..	..	..	..	+
21	+	..	..	..	..	..	..	..	0
24	..	..	..	..	+	..	..	..	+
24	0	0	0	0	0	0	0	0	+
27	..	..	+	..	..	..	..	..	+
30	..	..	..	..	..	+	..	+	+
30	..	..	..	..	..	..	..	+	..
35	..	..	+	..	+	..	..	..	+ Rabbit
	7	2	6	0	5	3	0	5	11
No Symptoms, Lost Weight									
10	+	..	+	..	..	..	..	+	0
10	+	+	+	+	+	..	..	..	0

0 = cultures not taken.

introduced into the blood stream. The work of the latter authors especially shows the important rôle played by the cells of the lungs, liver, spleen, and bonemarrow and the possibility of reinfection of the blood from foci in these organs. It is possible that the irregular findings

[35] Jour. Exper. Med., 1921, 33, p. 77.
[36] Jour. Gen. Physiol., 1921, 3, p. 465.
[37] Jour. Infect. Dis., 1920, 27, p. 527.
[38] Ibid., p. 327.
[39] Ibid., 1921, 28, p. 408.

in the heart blood of our experimental animals may be due to this cause. Barlett and Ozaki,[40] who have studied phagocytosis in vivo have shown that the cells of the liver and spleen act to a greater or less extent in a compensatory way for the deficiency of the leukocytes of the blood as they become less capable of ingesting bacteria. They believe this fact to be of great significance in eliminating bacteria from the blood. The fact that we have more often found B. Botulinus in these two organs than in the blood in fatal cases might in a measure corroborate this idea. The same result is also obtained in those cases in which latency of the spores occurs or at least in which symptoms from toxin formation are greatly delayed.

It is unfortunate that the brain was studied in only about 50% of our animals, as the findings for that organ cannot be fairly compared with those of a majority of the other organs. However, from the data at hand, the brain tissue in fatal cases seems to play a rôle second in importance only to that of the liver, or perhaps the spores or bacilli find conditions there more favorable for growth than in many of the other organs. The cultures of the bonemarrow and spleen appear to give equal results for all animals dying of botulism, though in the case of fed animals which presented no symptoms, the organism was found more frequently in the bonemarrow than in any other tissue except the lungs. Allowance must be made in the case of the latter organ for possible direct and continuous contamination from feces and food. The persistence of the spores in the feces of our fed animals which survive long periods is interesting. We have seen no evidence which would support the conclusion that toxin is formed in the intestinal tract in quantities sufficient to cause symptoms of botulism when, or if, absorbed into the blood stream.

Though several slightly pathogenic anaerobes of the putrefactive type as well as some of the highly pathogenic saccharolytic anaerobes, notably B. welchii and B. tetani, lead a semisaprophytic existence in the intestinal tract, the result of the work of this laboratory shows that the spores of B. botulinus are rarely found in the feces of normal animals and have not yet been found in the stools of healthy human beings; so that the possibility that the few spores thus encountered or even greatly increased numbers might produce toxin in the intestines is extremely unlikely. Reference to the data presented in tables 1 and 2 will show the enormous doses of spores even when fed in toxic solution that may be required to produce symptoms of botulism as against

[40] Jour. Med. Res., 1917-18, 37, p. 139.

the number injected subcutaneously which are required to obtain the same results. This would tend to support our contention. It is another matter when toxin-free spores are accidentally or experimentally ingested in prodigious numbers. They are then frequently introduced into an empty stomach in a concentrated mass and possibly are absorbed from points in the tract where they remain concentrated in the fluid and before any great dissemination throughout the tract has taken place. This is possibly a condition which facilitates their absorption, or at least they penetrate the intestinal wall in numbers too great to be destroyed by the natural defenses of the tissues, where the toxin is probably formed or liberated. Bronfenbrenner and Schlesinger [41] theorize as to points in the digestive tract from which the toxin of B. botulinus is probably absorbed. They have shown that the potency of the toxin is enormously increased in an acid medium and that in addition to its specificity it contains some substance capable of increasing the permeability of the intestine so as to permit the absorption of the specific toxin. Therefore, they believe that when it encounters the acid medium of the upper digestive tract (stomach and upper duodenum), it is readily absorbed. If that be so, it would seem that any toxin which passes this point or is formed in the lower tract might be neutralized, attenuated, or destroyed by the enzymes or alkaline metabolites produced by other bacteria, and that may be why such enormous numbers of detoxified spores, if they produce toxin at all, are innocuous when ingested.

Having found B. botulinus so persistently in the organs of our animals when fed or injected subcutaneously with the spores of this organism, we desired to ascertain if possible which organs were invaded first after the spores reached the blood stream. We, therefore, injected the spores intravenously as well as by other routes into a series of animals and killed them at varying intervals. Unfortunately the dose injected intravenously was so large that our results enumerated in table 10 enable us to form no definite conclusions on this subject.

It is evident that a comparatively small number of spores of B. botulinus fed to guinea-pigs do not invade any of the tissues, but the principal information gathered from this experiment proves that the same number of spores, when once in the blood stream, rapidly invade all the tissues of the body. We wish also to call attention to the fact that all the organs of 4 guinea-pigs in this series (2 cage checks and 2 fed) were negative for B. botulinus. This, taken in conjunction with

[41] Jour. Am. Med. Assn., 1922, 78, p. 1519.

the cultural studies of the organs of our other check animals, in which we have rarely found B. botulinus, should be taken as sufficient evidence that our cultural findings in these experiments are the result of our inoculations of spore suspensions.

Meyer, Neilson and Feusier [39] have shown that the distribution and destruction of small or large doses of typhoid bacilli are practically the same in normal and immunized rabbits; so that the question arose in our minds as to whether the neutralization of the toxin of B. botulinus

TABLE 10

RESULTS OF EXPERIMENTS WITH STRAINS 97, TYPE A AND 108, TYPE B MIXED CULTURE; PRACTICALLY NO RODS

No.	Weight	Spores	Symptoms	Survival, Hours	Killed or Died	Positive Organs	Remarks
				Jugular Vein			
131	336	Check	None	114	Killed	None	
132	350	20 M	None	18	Killed	All	
133	360	20 M	None	43	Killed	All	
134	344	20 M	None	46	Killed	All	
135	350	20 M	Botulism	67	Killed, very ill	All	
136	350	20 M	Botulism	70	Killed, very ill	All	
137	323	20 M	Botulism	60	Died	All	
138	356	20 M	Botulism	80	Died	All	
140	256	20 M	None	17	Killed	All except blood, brain, kidney, mes.	Blood coagulated, little taken
141	265	20 M	Botulism	69	Killed, very ill	All	
				Subcutaneous			
142	309	20 M	None	85	Killed	Liver, spleen, ing.	
143	315	20 M	None	87	Killed	All	
				Fed			
144	360	750 M	None	Survived			
145	265	20 M	None	95	Killed	None	
147	230	20 M	None	96	Killed	None	
146	200	Check	None	97	Killed	None	

as formed in these intravenous injections could have any influence on the distribution of this organism in the various tissues. We, therefore, inoculated 4 guinea-pigs with a similar dose of a different strain and 10 minutes later injected subcutaneously 2 of them with normal horse serum and 2 with antitoxin corresponding to the type of organism injected. We also endeavored to determine whether there was circulating toxin in the emulsified organs of any of these guinea-pigs, and, finally, whether the cultures of these organs were produced by spores

(and bacilli) or by bacilli alone. We found that the emulsified brain, spleen and liver of one guinea-pig (155) injected with antitoxin were toxic for mice in 5, 4 and 6 days, respectively, but the same organs of guinea-pig No. 153, receiving no antitoxin, were not toxic for mice. This result is difficult to interpret, and the experiment should be repeated on a larger scale before forming any conclusions. A microscopic study of the emulsions of the organs gave the following results: Rare spores and gram-positive bacilli were seen in preparations of the blood of No. 152, while the blood of No. 154 was negative. The spleen and liver of No. 153 showed rare gram-positive bacilli, resembling B. botulinus but no spores, while the same organs of guinea-pig 155 were negative. The blood of these last two animals was not examined microscopically. The other results obtained in this series are shown in table 11.

A study of this table shows, as in our previous experiment with intravenous injections, the rapid dissemination of the organism throughout the body. The failure to recover from the brain in most of these cultures is unusual. It is possible that this organ is the last to be invaded, and under the conditions of this experiment the animals died or were killed (except 155) before the organism had lodged in the brain. In experiments previously reported, we have frequently failed to recover the organism from the brains of animals dying within 48 hours. All heated culture tubes which proved sterile had been incubated from 13 to 15 days. It should be stated that as nearly equally large amounts of emulsion as possible, were inoculated into each tube. The outstanding feature of this experiment is the direct proof of germination of the spores of B. botulinus in the animal body, as shown by the failure to obtain cultures from those tubes which were heated, when the same emulsion in unheated tubes gave a positive culture. This occurred 6 times and shows conclusively that these organs had either been invaded by vegetating forms or that the spores which they might have contained had germinated. No great difference is apparent between the cultures of the organs of those guinea-pigs which did and those which did not receive antitoxin.

After this encouraging result, we attempted to demonstrate in an even more conclusive manner the germination and possible multiplication of these organisms in the animal body. A tied loop or pocket was formed in the jugular vein of 3 rabbits. One-half to 1 c c of emulsion containing from 50,000,000 to 250,000,000 freshly detoxified spores was injected with aseptic precautions into this tied vein. After 48 to 69

hours this portion of the vein was excised without disturbing its contents, and a drop of liquid from the vein stained by the Gram method. The vein and contents from 2 rabbits was ground up with sterile saline and injected into mice with fatal results in 6½ and 17 hours. Microscopic preparations of this emulsion, as well as from the contents of the

TABLE 11

RESULTS OF EXPERIMENTS WITH STRAIN 23, TYPE A—(FROM BROTH) RARE GRAM-NEGATIVE RODS

Serum Injections Subcutaneous

Intravenous					Positive Organ Emulsions				
No.	Weight	Spores	Symptoms	Survival			No. 152	No. 153	No. 154	No. 155
152	320	15 M + 0.5 c c N. serum	Botulism	Died after 45 hrs	Lungs	Heated	+	+	0	0
						Unheated	+	+	+	+
153	369	15 M + 0.5 c c N. serum	Botulism	Died after 70 hrs.	Kidney	Heated	—	—	0	0
						Unheated	+	+	+	—
154	388	15 M + 0.5 c c anti-toxin A	None	Killed after 47 hrs.	Mes.	Heated	+	+	0	0
						Unheated	+	+	+	+
					Ing.	Heated	—	+	0	0
155	389	15 M + 0.5 c c anti-toxin A	None	Killed after 69 hrs.		Unheated	+	+	+	+
					Bone	Heated	+	+	0	0
						Unheated	+	+	+	+
					Spleen	Heated	+	+	+	+
Subcutaneous						Unheated	+	+	+	+
					Liver	Heated	+	+	+	+
156	424	225 M	Botulism	Died after 6 days		Unheated	+	+	+	+
157	470	225 M	Botulism	Died after 7 days	Brain	Heated	—	—	—	—
						Unheated	—	0	—	+
					Blood	Heated	—	—	+	0
						Unheated	—*	+	+	+
					Gall	Heated	—	—	—	0
						Unheated	—	—	—	+

* Small amount inoculated; 0, cultures not taken.

vein alone from all rabbits showed rare spores and a definite growth of gram-positive organisms with division (7 to 10 per microscopic field) which morphologically resembled B. botulinus. These emulsions immediately inoculated in equal amounts into deep agar (heated and unheated) gave a luxurious growth of typical colonies of B. botulinus in the unheated tubes, while those that were heated showed only rare

colonies of the same organism. The rabbits died in from 3½ to 6 days after injection. The centrifugalized blood serum of all the rabbits was fatal to mice. The emulsion of bone marrow and spleen of 2 rabbits were tested and only the spleen of 1 rabbit proved sufficiently toxic to produce death in a mouse in 36 hours. We also attempted to produce a growth from the spores of B. botulinus in the anterior chamber of the eyes of 2 rabbits. The fluid from the eye of 1 rabbit, removed after 76 hours, showed only a few gram-positive bacilli with division, but no spores. The fluid from the eye of the other rabbit, at the end of 20 hours, while most of the spores and bacilli had been removed by the intense leukocytosis which prevailed, thus duplicating the phagocytosis in the peritoneal fluid, enough gram-positive bacilli with end to end forms remained to show a definite germination and multiplication of the B. botulinus spores injected. Inoculation of this fluid directly into agar gave a culture of B. botulinus contaminated only by a few colonies of cocci. The fluid from the eye of each rabbit was fatal for mice (table 12).

We consider the results of these last two experiments as giving definite and conclusive evidence that the detoxified spores of B. botulinus actually germinate and multiply in the animal body. To just what extent this multiplication continues, we are not prepared to express an opinion. In the rabbits intravenously injected, death may have been caused by the transudation of the toxin through the vein wall into the circulating blood, or as the tied vein was not completely stripped of its minute collateral branches some of the bacteria or toxin may have escaped into the blood stream through these small vessels and produced more toxin there. In the same way, it is possible to explain positive cultures for some of the organs. The culture of these spores in a tied vein simulates closely the culture in vitro. The circulation of the blood is prevented or is exceedingly sluggish and more nearly resembles that of the lymphatic and retarded peripheral circulation of the blood which may be favorable to the growth of bacteria. In all events the growth, especially in No. 3, was most conclusive.

DISCUSSION

Whether a few spores remaining latent in the intestinal tract could ever find conditions favorable to extensive growth and consequent toxin production or invasion of the tissues seems extremely unlikely and remains for further quantitative cultural studies to decide. From the

argument presented in considering the results of our cultural studies, it would appear that unless introduced in enormous numbers we have little to fear from the ingestion of toxin-free spores of B. botulinus, but we have shown that, when thus introduced, they cause symptoms and death from botulism. It has not yet been proved that the ingestion of small quantities of spores together with sublethal doses of toxin

TABLE 12

RESULTS OF EXPERIMENTS WITH STRAIN 23 — RARE VEGETATIVE FORMS; INJECTION OF HEATED SPORE SUSPENSION

Tied Vein of Rabbits

No.	Dose, C c	Amount	Vein Excised	Microscopic Vein Emulsion	Toxicity Vein	Survival, Days	Toxicity Organs for Mice	Cultures Organs
1	0.5	Approx. 50 M	69 hrs.	Rare spores, rare gram + bacilli division	.....	6	Blood serum +	Liver, blood neg.
2	0.75	Approx. 100 M	48 hrs.	Rare spores, rare gram + bacilli division	+ typical 17 hrs.	4	Blood serum + Bone, spleen —	Blood neg. Spleen, bone +
3	1.0	Approx. 250 M	66 hrs. agar culture heated + unheated ++++	Rare spores, good growth, gram + bacilli division	+ typical 6½ hrs.	3½	Blood + Bone — Spleen + 36 hrs.	Blood neg. Bone, heated — unheated + Spleen, heated + unheated +
Anterior Chamber of Eye								
4	..	Not counted	Fluid withdrawn 76 hrs.	No spores, rare gram + bacilli division	Eye fluid + 24 hrs.	7	Blood serum +	Eye fluid ++ Bone — Spleen, heated — unheated + Blood —
5	..	Not counted	20 hrs. agar culture heated + unheated ++++	Several spores, many gram + bacilli division	+ 28 hrs	2	Blood serum —	Eye fluid + Bone + Spleen + Liver + Blood —

can produce symptoms or death from botulism, but from our few experiments with toxic wash water containing few spores, taken in conjunction with the experimental evidence obtained by other workers [34] along these lines, it is quite likely that under these conditions they may invade the tissues, proliferate and produce enough additional toxin to cause symptoms of the disease. The fact that the organs of fatally infected animals are generally microscopically sterile is partial evidence that the toxin is produced from exceedingly few vegetative forms.

When we consider the potency of the toxin of B. botulinus, this need not surprise us. It is probable that the spores do not find conditions equally favorable for germination in all the tissues of the body and that the vegetative forms which arise from the few spores which survive the enzymes or phagocytes are, in turn, quickly destroyed and their toxin liberated in the process. This may be why we have been able to recover the organisms in some cases from so few of the tissues of animals which have succumbed to spore injections.

We have no theory to offer as to delayed infection by latent spores for in none of our experiments has this occurred. Neither have we, by the usual procedures for other bacterial species, been able to produce such an infection after several days in animals which had shown no symptoms. Undoubtedly the spores or bacilli of B. botulinus, possibly in small numbers, remained latent, particularly in the liver and bonemarrow, for considerable periods after the animals had received massive doses. Whether these latent organisms, after once being established in equilibrium with the fluids and tissues of the body ever germinate or multiply later in sufficient numbers to produce the disease, is more or less of an open question. We are aware that botulism has occurred in animals which had been fed several months previously with spores and maintained afterward in cages grossly contaminated with dejecta or foods containing spores of B. botulinus, but we cannot be sure in these rare cases that there may not have been a continuous reinfection of the lungs and intestinal tract from the feces or food remaining in the cage. We have seen 1 normal animal in such a cage die of symptoms suspiciously resembling those of botulism.

Why some animals should succumb to botulism and others of the same weight receiving the same large dose under identical conditions should fail to show symptoms is another question which we have not attempted to answer. A natural immunity probably does not exist except under the conditions of the experiments, for we have later killed some of these surviving guinea-pigs with small doses of toxin. Neither in this case is it a question of the toxin having caused the germination or multiplication of latent spores or bacilli, for the normal controls died within the same period.

The mode of action or the paths of diffusion of the toxin of B. botulinus in the animal body has received no attention in our experiments, so that we have not sufficient information to discuss this phase of

the question. Nor are we able to venture an opinion as to which of the tissues may play a preponderating rôle in the neutralization of the toxin as produced. The fact that emulsions of the organs of animals dying of botulism are so infrequently toxic leads us to suppose that the amount of toxin circulating in the system, at least postmortem, is very minute. It may be that at the time that the symptoms were produced and the damage done to the system it was in greater quantity and had been subsequently attenuated or destroyed by fixation in the tissues, or the process of this fixation may have been the condition causing the manifestation of symptoms.

While it is remotely possible, though we have no evidence to support the belief, that some of the toxin liberated in the animal body may be derived from a preformed endotoxin within the heated spore or bacterial cell, we have demonstrated beyond doubt that the toxin-free spores of B. botulinus do germinate and multiply in the fluids and tissues of the animal body and produce toxin there. Our cultural studies would tend to substantiate these conclusions. Therefore, in our opinion, the principal, if not the only, source of toxin in the body of our experimental animals is from the germination of these spores.

We wish to say in conclusion that our results showing the difficulty of infecting laboratory animals except by massive doses of heated B. botulinus spores should not be taken in any way as a criterion for what may happen in the human system by the consumption of spores of this organism in the natural way—although undoubtedly in the latter case enormous numbers if freed of their toxin would be required to produce infection.

CONCLUSIONS

Massive doses of toxin-free spores of B. botulinus are pathogenic when introduced into the animal body.

These spores and the vegetative forms arising from them are rapidly disseminated throughout the tissues of the body.

Toxin-free spores of B. botulinus germinate, and the vegetative forms arising from this germination multiply and liberate toxin in the animal body.